Protection against Renal Ischemia Reperfusion Injury by CD44 Disruption

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Inflammation contributes to renal ischemia reperfusion (I/R) injury, potentially causing renal dysfunction. The inflammatory infiltrate mainly consists of neutrophils, which are deleterious for the renal tissue. Because CD44 is expressed by neutrophils and is rapidly upregulated by capillary endothelial cells after I/R injury, it was hypothesized that CD44 might play an important role in the development of I/R injury. This study showed that rapid CD44 upregulation on renal capillary endothelial cells mediates neutrophil recruitment to the postischemic tissue. Hence, CD44 deficiency led to decreased influx of neutrophils regardless of comparable levels in chemotactic factors expressed in the kidney. The reduced influx of neutrophils was associated with preserved renal function and morphology. Adoptive transfer experiments of labeled neutrophils revealed that endothelial CD44 rather than neutrophil CD44 mediates neutrophil migration. Activation of neutrophils increased cell-surface expression of hyaluronic acid (HA). Altogether, a novel mechanism in the recruitment of neutrophils that involves interaction of endothelial CD44 and neutrophil HA was found. Either blocking endothelial CD44 or removal of neutrophil HA decreased rolling and adhesion of neutrophils. Administration of anti-CD44 to mice reduced the influx of neutrophils into the postischemic tissue, associated with renal function preservation. Therefore, anti-CD44–based therapies may contribute to prevent or reduce renal I/R injury.


Renal injury as a result of ischemia reperfusion (I/R) is a major clinical problem and is the most common cause of acute renal failure after renal transplantation, shock, sepsis, and renal artery stenosis. For patients in shock, acute tubular necrosis is associated with a mortality rate of approximately 50%, and little has changed in the past 40 yr (1). Treatment of I/R injury is still only supportive. Therefore, development of therapeutic interventions to prevent or reduce renal tissue injury after I/R remains the focus of research.

CD44 is a family of type I transmembrane glycoproteins with a wide tissue distribution, including expression on leukocytes and epithelial and endothelial cells. CD44 family glycoproteins are encoded by a single gene that consists of 19 exons. By alternative splicing, different isoforms are generated (2–5). These isoforms have been implicated in many physiologic and pathologic processes, such as cell–cell and cell–matrix interaction, leukocyte extravasation, wound healing/scarring, cell migration, lymphocyte activation, and binding/presentation of growth factors (6–9). All isoforms contain a hyaluronic acid (HA) and osteopontin binding site, which are the major ligands of CD44 (7,10).

Little is known about the physiologic role of CD44 in the kidney after renal injury. Under normal conditions, CD44 is hardly expressed in the kidney except in passenger leukocytes. In inflammatory renal diseases, CD44 expression is markedly enhanced in glomeruli and on injured renal tubular epithelial cells and capillary endothelial cells, as documented in both human (11,12) and several animal models of renal diseases (13–16). Within 1 d after renal I/R injury, CD44 expression is observed on infiltrating leukocytes and capillary endothelial cells (17).

I/R injury is characterized by a massive influx of neutrophils early after reperfusion, which exert a crucial role in the pathophysiology of postischemic renal failure by the release of cytotoxic proteases and oxygen-derived radicals (18). Attraction of neutrophils to the postischemic tissue is regulated by endothelial adhesion molecules, complement components such as C5a, and a number of chemokines such as IL-8 (murine homologue is keratinocyte-derived chemokine [KC]) and macrophage inflammatory protein-2 (MIP-2). We hypothesized that CD44 plays a major role in the massive influx of neutrophils and the subsequent damage to the renal tissue after I/R.

Here we report that CD44 significantly contributes to the migration of neutrophils into the postischemic renal tissue and to the development of renal damage and subsequent renal failure. We describe a novel mechanism in which CD44 expressed on endothelial cells interacts with HA on neutrophils, leading to recruitment of neutrophils to the site of inflammation. In addition, we show that disruption of CD44–HA inter-
actions is effective and offers a new therapeutic target for prevention of I/R injury.

Materials and Methods
Mice and Experimental Protocol
Bilateral ischemia or sham surgery was performed under general anesthesia (0.07 ml/10 g mouse of FFM mixture, containing 1.25 mg/ml midazolam [Roche, Mijdrecht, The Netherlands], 0.08 mg/ml fentanyl citrate, and 2.5 mg/ml fentanyl [Janssen Pharmaceutica, Beerse, Belgium]) in 6- to 8-wk-old male mice. CD44 knockout on C57Bl/6 background (CD44−/−) (19) and C57Bl/6 wild-type (CD44+/+) mice were bred in our animal facility. Antibody-treated mice received 16 h before surgery a single intraperitoneal injection of 100 μg of anti-CD44 mAb (6,20) (clone IM7.8.1, rat IgG2b; ATCC, Livermore, CA; purified by protein G-Sepharose chromatography [Calbiochem, Darmstadt, Germany]) that interacts with the HA-binding site of CD44 (21) and may induce shedding of CD44 (22), or control IgG (purified rat IgG2a; Pharmingen, Erembodegem, Belgium). Renal arteries were bilaterally occluded for 45 min with nontraumatic microvascular clamps, during which the mice were placed in a 32°C incubator. All mice received postoperative analgesia (0.15 mg/kg buprenorphine, subcutaneously [Shering-Plough, Brussels, Belgium]). Sham-operated mice underwent the same procedure without clamping of the arteries and were killed 1 d after surgery. Groups of mice (n = 8) were killed 1, 3, 7, and 14 d after surgery by exsanguination under general anesthesia. All experimental procedures were approved by the Animal Care and Use Committee of the University of Amsterdam, The Netherlands.

Histology, Immunohistochemistry, and Renal Function
Renal tissues were fixed in 10% formalin for 12 h and embedded in paraffin. Sections were cut, deparaffinized, diastase treated, and stained with periodic acid-Schiff. For immunostaining, specific antibodies were used: CD44 (clone IM7.8.1; Dianova, Hamburg, Germany), and HA (biotinylated HA-binding protein; Calbiochem). As a negative control, we used species- and isotype-matched antibodies. Serum urea and serum creatinine were determined using a standard enzymatic method (Boehringer Mannheim, Indianapolis, IN). Histopathologic scoring

Flow Cytometric Analysis of HA on Human and Murine Neutrophils
Heparin blood was withdrawn as described above. Erythrocytes were subjected to hypotonic lysis. Leukocytes were incubated with biotinylated HA-binding protein (Calbiochem) for 30 min at room temperature. Bound HA-binding protein was visualized using FITC-conjugated streptavidin (DAKO, Glostrup, Denmark). Pretreatment of leukocytes with 300 μg/ml hyaluronidase type IV-s (Sigma, St. Louis, MO) for 10 min prevented binding of HA-binding protein. Whole-blood stimulation with LPS (United States Pharmacopeial Convention, Rockville, MD) or formyl-Met-Leu-Phe (fmlp; Sigma Chemicals) was performed for 30 min at 37°C. Murine neutrophils were selected by additional Ly-6G-PE (Pharmingen) staining. Human neutrophils were gated in a FSC-SSC plot. Expression of HA was analyzed in the FL-1-channel.

Endothelial Cells
Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins as described (23). Cells were cultured in RPMI 1640 supplemented with 20% (vol/vol) human serum, 200 μg/ml penicillin, and streptomycin (Life Technologies, Breda, The Netherlands) until confluence in 5 to 7 d. Rolling experiments under flow conditions were performed with endothelial cells of the second or third passage. HUVEC monolayers were activated by TNF-α (100 U/ml, 6 h, 37°C) before the rolling experiments.

Neutrophil Isolation and Pretreatment in Rolling Assays
Human neutrophils were isolated using Ficoll-Paque (Amersham Pharmacia Biotech, Freiburg, Germany), according to the manufacturer’s instructions. Erythrocytes were subjected to hypotonic lysis. The HA-binding site of CD44 on endothelial cells was blocked using anti-CD44, clone BU75 (AnceIl, Bayport, MN), which is reported to specifically inhibit HA binding to CD44 (24). As control, a matched mouse IgG2a was used (clone RPC 5; Ancell). For removing HA from the cellular surface of neutrophils, neutrophils were incubated in a phosphate-buffered solution (pH 7.0) that contained 300 μg/ml hyaluronidase type IV-s (Sigma) for 10 min. Control cells were incubated in the identical buffer without hyaluronidase.

Perfusion experiments were performed as described previously (25). In short, 1 million neutrophils were perfused through a HUVEC monolayer-containing chamber. Video images were analyzed using the image analysis software Optimas 6.1 (Media Cybernetics Systems, Silver Spring, MD). Approximately 200 neutrophils, in contact with the HUVEC, were analyzed. The results were obtained using blood of three different healthy controls in three independent experiments.

Statistical Analyses
All data were analyzed by unpaired t test comparison. Multiple comparisons were performed using a repeated measures ANOVA corrected by a Bonferroni post hoc test.
Results
Renal CD44 Expression after I/R Injury

One day after I/R, CD44 expression was detected on peritubular endothelium and infiltrating cells in the interstitium (Figure 1A). Three, 7, and 14 d after I/R, CD44 expression increased further, and, besides leukocytes and endothelial cells, CD44 was detected laterally on tubular epithelial cells in the corticomedullary region. In sham-operated mice, renal CD44 expression was restricted to passenger leukocytes (Figure 1D).

CD44 Deficiency Preserved Renal Function and Decreased Renal Injury

To study the role of CD44 in I/R injury, we subjected CD44+/+ and CD44−/− to bilateral I/R and compared renal function. Twenty-four hours after I/R, the increase in serum creatinine and urea levels was significantly prevented in CD44−/− (Figure 2, A and B). Throughout the recovery phase, renal function of CD44−/− was improved by approximately 50% compared with CD44+/+. In addition, renal function was fully restored after 14 d in CD44−/−, whereas renal function of CD44+/+ was still impaired. In accordance with renal function, histopathologic changes in the corticomedullary region, such as tubular necrosis, dilation, and loss of brush border, were reduced in CD44−/− mice (Figure 2C).

CD44 Deficiency Diminished Neutrophil and Macrophage Infiltration

Because neutrophils exert a crucial role in the development of postischemic renal injury, we evaluated their influx in CD44+/+ and CD44−/− after I/R. As described previously (19), comparison of peripheral blood leukocytes revealed no differences in total leukocyte (P > 0.6), neutrophil (P > 0.9), lymphocyte (P > 0.5), or monocyte (P > 0.8) counts between CD44+/+ and CD44−/−. As illustrated in Figure 3A, CD44 deficiency greatly reduced the influx of neutrophils into the postischemic kidney 1 d after I/R. Reduced neutrophil influx (P < 0.01) was confirmed by flow cytometric analysis in CD44−/− (0.37 ± 0.08% of total cells) and CD44−/− (0.16 ± 0.06% of total cells). However, the number of neutrophils 3 and 7 d after I/R was increased in CD44−/− compared with CD44+/+, suggesting impaired clearance of neutrophils in the absence of CD44.

Influx of macrophages in postischemic CD44−/− kidneys was decreased at all time points compared with CD44+/+ (Figure 3B). To determine whether the difference in influx of neutrophils was mediated through differences in expression of chemokine/cytokine levels, we assessed homogenates of CD44+/+ and CD44−/− postischemic kidneys for KC, MIP-2, and IL-1β (Figure 4). Although KC, MIP-2, and IL-1β levels were elevated at days 1 and 3 after ischemia, no differences between CD44+/+ and CD44−/− that could explain the re-

Figure 1. De novo CD44 expression after renal ischemia reperfusion (I/R). Representative immunostainings for pan-CD44 of CD44+/+ kidneys 1 d (A), 7 d (B), or 14 d (C) after I/R or sham-operated (D) animals. Representative for n = 8; arrows indicate CD44-positive staining on capillary endothelial cells. Magnification, ×25 in A through D; ×100 insert in A.
duced neutrophil influx in CD44−/− postischemic kidneys were detected.

**HA and Osteopontin Expression in CD44−/− and CD44+/+ after I/R**

Because HA is one of the principal ligands of CD44 and promotes inflammation, we analyzed HA expression in postischemic kidneys by immunohistochemistry. Interstitial HA-positive areas expanded in the postischemic kidneys of both genotypes (Figure 5A). Initially and at days 1 and 3 after I/R, no significant difference in HA expression was detected between CD44+/+ and CD44−/−, yet clearance of HA fragments was reduced in CD44−/− at days 7 and 14 after I/R.

Expression of the second major ligand of CD44, osteopontin, was increased after I/R injury (Figure 5B). However, no differences in osteopontin expression were observed between CD44+/+ and CD44−/−.

**Neutrophil Influx Is Mediated by Renal CD44 Rather Than Neutrophil CD44**

To determine whether neutrophil bound CD44 or renal CD44 mediated neutrophil influx into the postischemic tissue, we performed adoptive transfer of neutrophils. A mixture of labeled CD44+/+ and CD44−/− neutrophils was injected into recipient mice. Adoptive transfer of CD44+/+ and CD44−/− neutrophils in CD44+/+ recipients revealed no differences in migration of neutrophils into the postischemic kidney (Figure 6). In contrast, migration of both CD44+/+ and CD44−/− neutrophils was impaired in CD44−/− postischemic kidneys compared with CD44+/+ in relative (Figure 6A) as well as in...
absolute quantifications (Figure 6B). This suggested that interaction of CD44 expressed by postischemic renal cells mediated adhesion and migration of neutrophils. No circulating labeled neutrophils could be detected after 24 h of reperfusion in the blood. As shown in Figure 1A, CD44 is rapidly upregulated on peritubular capillary endothelial cells. Therefore, it is most likely that endothelial CD44 mediates neutrophil influx into the postischemic kidneys.

**HA Is Expressed by Neutrophils**

We evaluated potential expression of CD44 ligands by neutrophils. Flow cytometric analysis revealed membrane-bound HA, the major CD44 ligand, on murine neutrophils. Pretreatment of the neutrophils with hyaluronidase reduced binding of the HA-binding protein (Figure 7A). No difference could be detected in expression of HA on CD44<sup>−/−</sup> and CD44<sup>+/+</sup> neutrophils (data not shown). In addition, HA expression was detected on human neutrophils, which could be fully degraded by hyaluronidase pretreatment (Figure 7B). Whole-blood activation with fmlp led to a dose-dependent upregulation of HA on neutrophils (Figure 7C).

**Endothelial CD44 and Neutrophil HA Are Involved in Rolling and Adhesion of Neutrophils**

Our data strongly suggest that neutrophil extravasation into posts ischemic kidneys occurs through the interaction of HA on neutrophils (Figure 7) and CD44<sup>−/−</sup> endothelial cells (Figures 1A and 6). To confirm these findings, we performed rolling assays under flow conditions with neutrophils on HUVEC. Blocking endothelial CD44 with an anti-CD44 that specifically inhibits HA binding (24) decreased rolling of neutrophils (Fig-
ure 8A) and increased their rolling velocity (Figure 8B). This resulted in diminished numbers of adhering cells to the endothelial cells (Figure 8C). Furthermore, removal of HA from the cell surface of neutrophils by hyaluronidase treatment had similar effects on rolling (Figure 8A), rolling velocity (Figure 8B), and adhesion (Figure 8C) of neutrophils on activated HUVEC.

Blockade of CD44–HA Interactions Reduced Neutrophil Influx and Subsequently Protected Renal Function

Administration of an antibody that interacts with the HA-binding site of CD44 (21) and may induce shedding of CD44 (22) decreased neutrophil migration into the postischemic kidney (Figure 9A). Injection of the antibody did not alter peripheral blood leukocyte numbers ($P > 0.6$) or neutrophil ($P > 0.3$), lymphocyte ($P > 0.4$), or monocyte ($P > 0.2$) counts. In accordance with neutrophil influx, renal function, as determined by serum creatinine and urea, was protected in anti-CD44–treated animals compared with the control antibody–treated animals (Figure 9B).

Discussion

It has generally been recognized that massive influx of neutrophils is a major mediator in the development of postischemic renal failure through the release of cytotoxic proteases and oxygen-derived radicals. In this study, we show that CD44 exerts a crucial role in the migration of neutrophils into the postischemic tissue. CD44 deficiency or CD44 blockade was shown to decrease neutrophil influx, reduce renal injury, and preserve renal function.

First, we show that CD44 is rapidly upregulated in postischemic kidneys. Early after I/R injury, its expression is found predominantly on infiltrating leukocytes and capillary endothelial cells, as described earlier by Lewington et al. (17). After 3 d of I/R injury, CD44 expression is also found laterally on tubular epithelial cells, which is in agreement with previous studies reporting CD44 expression in various models of kidney diseases (13–16) and human nephropathies (11,12).

Second, we demonstrate that lack of CD44 decreases renal
injury after I/R and preserves renal function. I/R injury is characterized by the influx of neutrophils into the postischemic tissue, exerting a deleterious role in the pathophysiology of postischemic renal failure by the release of cytotoxic proteases and oxygen-derived radicals (18). In accordance, depletion of neutrophils decreased renal injury (26). In our model, we observed a >50% decrease in neutrophil influx in CD44-deficient mice. These data are in contrast with other animal models, including tuberculosis (27,28), Escherichia coli pneumonia (29), and bleomycin-induced pulmonary fibrosis (30), in which CD44 deficiency leads to increased neutrophil levels. This suggests that different pathogenic mechanisms in these pulmonary diseases are at play. In the lungs, resident alveolar macrophages seem to be the major source of cytokines and chemokines. Furthermore, the model of I/R is an acute type of injury in which the infiltration of neutrophils is limited to 1 d, in contrast to the models of chronic pulmonary injury. Three and 7 d after I/R, most neutrophils are cleared from the injured kidneys in CD44+/+ mice, as expected from the literature. In contrast, the resolution of inflammation in the CD44−/− postischemic kidney is delayed probably as a consequence of reduced macrophage influx, impaired clearance of inflammatory cell remnants (31), and decreased apoptosis of neutrophils (32) in the absence of CD44. After I/R, the number of neutrophils is 30-fold higher than the number of macrophages, suggesting a relatively small contribution for macrophages in renal I/R injury. Furthermore, the differences in macrophage presence in CD44+/+ and CD44−/− are unlikely to exert great impact on development of renal damage and recovery of renal function (33). Furthermore, Teder et al. (30) described that resolution of inflammation is impaired in the absence of CD44 as a result of reduced HA clearance. Our data are in agreement with these findings. Because HA has well-known proinflammatory properties, impaired clearance of HA therefore may account for the higher numbers of neutrophils found at days 3 and 7 in the CD44−/− postischemic kidneys.

The second CD44 ligand, osteopontin, is a chemotactic factor for macrophages (10). Although in our study osteopontin expression in CD44+/+ and CD44−/− was comparable after I/R, abrogation of osteopontin-CD44 signaling may be responsible for reduced influx of macrophages in CD44−/− after I/R (33).

Third, to elucidate the mechanisms underlying the significant reduction in neutrophil influx after I/R, we measured cytokine and chemokine levels in postischemic kidneys. Indeed, a vari-

Figure 7. Flow-cytometric analysis of neutrophils revealed HA expression on the cellular surface. Murine (A) and human (B) neutrophils were probed with biotinylated HA-binding protein (HABP) or without (control) and visualized with FITC-labeled streptavidin. Hyaluronidase pretreatment of leukocytes prevented HA-binding protein binding to neutrophils. Whole-blood stimulation with fmlp increased the expression of HA on neutrophils in a dose-dependent manner (C). Human whole blood was stimulated without (control), with 10−7 M, or with 10−6 M fmlp.
ety of cells in the kidney are able to secrete cytokines and CXC chemokines, including tubular epithelial cells, monocytes, and mesangial cells. Upregulation of the CXC chemokines KC and MIP-2 has been reported after renal I/R injury (34). Expression of IL-1β increases the expression of adhesion molecules, such as intercellular adhesion molecule and vascular cell adhesion molecule. In cerebral I/R (35), CD44 deficiency reduced tissue damage and resulted in preservation of motor function as a result of lower levels of IL-1β. In agreement with previous studies, we detected upregulation of KC, MIP-2, and IL-1β after I/R, but no differences between CD44+/− and CD44−/− that could explain the differences in neutrophil influx were observed. Therefore, we hypothesized that CD44 may play a direct role in attraction, adhesion, or migration of neutrophils.

DeGrendele et al. (36,37) showed that extravasation of lymphocytes from the blood into the vascular wall is dependent on interactions of CD44 on the cell surface of lymphocytes (7,36,38), with HA on the endothelial cell surface bound to receptor for hyaluronan-mediated motility (RHAMM) (39) or CD44 (40). To test whether this mechanism may be at play in renal I/R, we performed adoptive transfer of CD44+/− and CD44−/− neutrophils before I/R. In contrast to the mechanism proposed by DeGrendele et al. (36,37) for lymphocytes, it was not neutrophil-bound CD44 that mediated migration of leukocytes into the postischemic tissue but renal expression of CD44, most likely by postischemic renal capillary endothelial cells. These results underline the differences between migration of lymphoid cells and neutrophils. Khan et al. (41) observed in chimeric mice, after intrascrotal injection of MIP-2, that CD44 on the endothelium as well as on the neutrophil was required to obtain optimal neutrophil emigration. This observation may
be tissue specific, because disruption of CD44 leads to increased emigration of neutrophils in several pulmonary models, e.g., tuberculosis (28), E. coli–induced pneumonia (29), and bleomycin-induced pulmonary fibrosis (30), and in arthritis emigration of neutrophils is CD44 independent (42). The involvement of CD44 in migration of neutrophils in extracellular matrix may vary according to the components of the matrix, for example, HA contents (29). The demonstration of HA at the cell surface of murine neutrophils is an important new finding of our study. Although HA is the major ligand of CD44, we could not detect HA contents (29). The demonstration of HA at the cell surface of neutrophils by non–CD44-dependent HA binding molecules, such as RHAMM (39). The finding that HA is also expressed on human neutrophils suggests an interspecies conserved mechanism. Neutrophil activation by fmlp stimulation but not LPS could increase the expression of HA on the cellular surface of neutrophils in a dose-dependent manner. This suggests a specific mechanism leading to upregulation of HA after neutrophil activation, thereby facilitating rolling and subsequent adhesion of neutrophils.

Altogether, our data suggest that interaction between endothelial CD44 and neutrophil HA plays a major role in extravasation of neutrophils into postischemic kidneys. Rolling experiments with TNF-α–stimulated endothelial cells confirmed the important role of endothelial CD44 and neutrophil HA in the rolling of neutrophils. In fact, either blocking the HA-binding domain of CD44 on endothelial cells or removal of HA from the neutrophils decreased rolling of these neutrophils by >50%. The study of Khan et al. (41) showed that rolling of leukocytes in vivo was not impaired in the absence of CD44. This suggests that CD44 plays a specific role in the rolling of neutrophils (10 to 25% of the total number of leukocytes in mice). The effect of CD44 may remain unnoticed when analyzed in an unfractin-ated pool of leukocytes (75 to 90% are monocytes and/or lymphocytes). The transition of rolling to adhesion is dependent on the time when the endothelial cell and neutrophil interact (43). In the performed perfusion experiments, disruption of the interaction between endothelial CD44 and neutrophil HA increased rolling velocity of neutrophils by >200%. Decreased numbers of rolling cells and increased rolling velocity resulted in adherence of fewer neutrophils (30%) to the endothelium. Using chimeric mice, Khan et al. (41) also showed that adhesion of neutrophils is dependent on endothelial CD44 rather than on neutrophil CD44.

Finally, we postulated that in the normal situation, neutrophils stay clear from the endothelium that is negative for CD44 and remain in the circulation. The de novo expression of CD44 on the capillary endothelial cells upon I/R mediates neutrophil adhesion by interaction with neutrophil-bound HA. Activation of neutrophils leads to increased presentation of HA, thereby increasing the interaction with endothelial CD44. The CD44–HA interaction results in a massive infiltration of neutrophils into the injured renal tissue.

To determine whether our results could be valid for therapeutic application and to exclude adaptive mechanisms in CD44−/−, we confirmed our results using an antibody that binds to the HA-binding region of CD44 (21) and may induce shedding of CD44 (22). Administration of the antibody to mice before ischemia resulted in a dramatic reduction of neutrophil influx into the postischemic kidney, diminished renal damage, and preserved renal function.

In conclusion, upregulation of CD44 on capillary endothelial cells after I/R injury results in the recruitment of neutrophils into the postischemic tissue through interactions with HA at the surface of neutrophils. CD44 deficiency or inhibition of CD44–HA ligation resulted in decreased influx of neutrophils and subsequent preservation of renal function. Therefore, targeting CD44–HA interactions may be an innovative and efficient therapy to prevent or reduce I/R related injury.

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